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Published in:
Biochemical and Biophysical Research Communications

Publication date:
1988

Dictyostelium discoideum mutant *synag 7* with altered G-protein–adenylate cyclase interaction

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Summary

Previous results have shown that *Dictyostelium discoideum* mutant *synag 7* is defective in the regulation of adenylate cyclase by receptor agonists *in vivo* and by GTPγS *in vitro*; the guanine nucleotide activation of adenylate cyclase is restored by the high-speed supernatant from wild-type cells.

Here we report that in *synag 7* membranes: (1) cyclic AMP receptors had normal levels and were regulated by guanine nucleotides as in wild-type; (2) GTP binding and high-affinity GTPase were reduced but still stimulated by cyclic AMP; (3) the supernatant from wild-type cells restored GTP binding to membranes of this mutant, and partly restored high-affinity GTPase activity; (4) the supernatant of *synag 7* was ineffective in these reconstitutions and did not influence GTP binding and GTPase activities in mutant or wild-type membranes.

These results suggest that the defect in mutant *synag 7* is located between G-protein and adenylate cyclase, and not between receptor and G-protein. A factor in the supernatant is absent in *synag 7* and appears to be essential for normal GTP binding, GTPase and activation of adenylate cyclase. This soluble heat-labile factor may represent a new molecule required for receptor- and G-protein-mediated activation of adenylate cyclase.

Key words: *Dictyostelium*, G-protein, adenylate cyclase.

Introduction

The receptor-linked adenylate cyclase system in *Dictyostelium discoideum* provides a useful model for comparison with the hormone and neurotransmitter-regulated adenylate cyclase systems in vertebrates. In the cellular slime mould *D. discoideum*, cyclic AMP functions as a hormone-like signal during chemotaxis (Konijn, 1970), morphogenesis (Schaap *et al.* 1984), and cell differentiation (Kay, 1982). Extracellular cyclic AMP is detected by cell surface cyclic AMP receptors (Devreotes, 1983), and results in several intracellular responses, including the activation of guanylate and adenylate cyclase (Gerisch, 1987; Janssens & Van Haastert, 1987), phospholipase C (Europe-Finner & Newell, 1987), and the phosphorylation of cyclic AMP receptors (Klein *et al.* 1985, 1987). Intracellular cyclic GMP reaches a peak at 10 s after stimulation and is possibly involved in cyclic AMP-induced chemotactic movement. Adenylate cyclase activity increases at about 10–30 s after cyclic AMP addition, and reaches a maximal level at 60–120 s. The cyclic AMP produced is secreted, and triggers more distal cells, thus relaying the signal (Van Haastert, 1984*a*). Surrounding cells respond to the cyclic AMP signal by chemotaxis and by secreting a pulse of cyclic AMP themselves (see reviews, Devreotes, 1983; Gerisch, 1987; Janssens & Van Haastert, 1987).

Several lines of evidence in *D. discoideum* have previously suggested that surface cyclic AMP receptors are coupled to intracellular effectors *via* G-proteins. Binding of cyclic AMP is complex, showing interconversions of binding states *in vivo* (Van Haastert & De Wit, 1984; Van Haastert, 1985; Van Haastert *et al.* 1986), which are promoted by guanine nucleotides *in vitro* (Van Haastert, 1984*b*; Van Haastert *et al.* 1986). Alternatively, cyclic AMP increases [^{3}H]GTP binding to isolated membranes, at the same time accelerating the dissociation rate of GTP (De Wit & Snaar-Jagalska, 1985). In addition, receptor agonists stimulate high-affinity GTPase activity (Snaar-Jagalska *et al.* 1988*b*). Recently, it has been shown that GTP stimulates the formation of *myo*-inositol 1,4,5-trisphosphate (Europe-Finner & Newell, 1987). Also adenylate cyclase can be stimulated (Theibert & Devreotes, 1986), and inhibited (Van Haastert *et al.* 1987) by GTP. However, stimulation by GTP and its analogues can only be observed in cell homogenates shortly after lysis, or in membranes at temperatures between 0 and 10 °C and in the presence of a cytosolic factor (Theibert & Devreotes, 1986). Preincubation of membranes under phosphorylation conditions converted

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stimulation of adenylate cyclase by guanine nucleotides to inhibition (Van Haastert *et al.* 1987). Inhibition of adenylate cyclase by GTP is blocked by pretreatment of cells with pertussis toxin (Van Haastert *et al.* 1987). Moreover, desensitization to persistent cyclic AMP stimuli is blocked after treatment with pertussis toxin *in vivo* (Snaar-Jagalska & Van Haastert, unpublished results). These observations suggest that adenylate cyclase regulation by G-protein, while present in *D. discoideum*, might operate somewhat differently from the way it does in vertebrates.

In vertebrate cells, surface receptor-linked adenylate cyclase systems were successfully investigated in S49 lymphoma cells, deficient or defective in stimulated G-protein (Gs). An aggregation-defective mutant of *D. discoideum* was isolated (Frantz, 1980). In this mutant, designated *synag 7* or N7, cyclic AMP and/or guanine nucleotides do not activate adenylate cyclase (Theibert & Devreotes, 1986; Van Haastert *et al.* 1987). The wild-type pattern of guanosine nucleotide regulation was restored by the addition of a high-speed supernatant from wild-type cells. In the present study the interaction of surface cyclic AMP receptors with G-proteins was investigated in membranes from the mutant and in the presence of wild-type supernatant. Addition of this supernatant restored the low GTP binding and GTPase activity in *synag 7* membranes. These results suggest that the supernatant factor absent in the mutant may represent a new molecule required for receptor- and G-protein-mediated activation of adenylate cyclase.

Materials and methods

Materials

[2,8-^{3}H]cyclic AMP (1·5 TBq mmol^{-1}), [8-^{3}H]GTP (sodium salt; 10·6 Ci mmol^{-1}) were obtained from Amersham. [γ-^{32}P]GTP (37·94 Ci mmol^{-1}) was purchased from New England Nuclear. cyclic AMP, ATP, ATPγS, AppNHp, GTP, creatine phosphate and creatine kinase were obtained from Boehringer-Mannheim. Dithiothreitol (DTT) and bovine serum albumin (BSA) were from Sigma.

Culture conditions and membrane isolation

The *synag 7* mutant of wild-type *D. discoideum* NC-4 has been characterized by Dr Frantz (1980) and was kindly provided by Dr P. N. Devreotes. Wild-type and mutant cells were grown as described (Van Haastert & Van der Heijden, 1983), harvested in 10 mM-Na/K phosphate buffer (PB), pH 6·5, washed and starved in PB by shaking at a density of 10^7 cells ml^{-1}. During starvation of mutant cells pulses of cyclic AMP were given at 6-min intervals and at a concentration of 10^{-7} M. After 5–6 h, cells were collected by centrifugation, washed twice with PB, and the pellet was resuspended in 40 mM-Hepes/NaOH, 0·5 mM-EDTA, 250 mM-sucrose, pH 7·7, to a density of 2×10^8 cells ml^{-1}. Crude membranes were prepared by pressing the cell suspension through a Nuclepore filter (pore size 3 μM) at 0°C; if it is not otherwise indicated, the membranes were washed once and finally resuspended in PB to the equivalent of 1×10^8 cells ml^{-1}.

Cyclic AMP binding

The association of [^{3}H]cyclic AMP with *D. discoideum* cells at 20°C was detected in a volume of 100 μl containing PB, 10 mM-DTT, 30 nM-[^{3}H]cyclic AMP, and 8×10^6 cells. At the times indicated the cells were separated from the extracellular medium by centrifugation through silicon oil as described (Van Haastert & De Wit, 1984*c*). The dissociation of bound [^{3}H]cyclic AMP was measured after equilibration of cells with 2 nM-[^{3}H]cyclic AMP for 45 s at 20°C. Dissociation at t = 0 was induced by the addition of 0·1 mM-cyclic AMP. Inhibition of cyclic AMP binding to membranes was measured at 20°C in an incubation volume of 100 μl containing 80 μl membranes, 2 nM-[^{3}H]cyclic AMP and different concentrations of guanine nucleotides. The incubation period was 75 s, followed by centrifugation through silicon oil. Non-specific binding was determined by including 0·1 mM-cyclic AMP in the incubation mixture and was subtracted from all data shown.

GTP binding

Binding of [^{3}H]GTP to membranes was measured at 0°C in an incubation volume of 100 μl containing 10 mM-Tris·HCl, pH 8·0, 100 nM-[^{3}H]GTP, 1 mM-ATP, 5 mM-$MgCl_2$, and 80 μl membranes. Bound [^{3}H]GTP was separated from free [^{3}H]GTP by centrifugation through silicon oil at 10 000 ***g*** for 30 s. Non-specific binding was determined in the presence of 0·1 mM unlabelled GTP.

GTPase assay

GTPase was determined as described (Snaar-Jagalska *et al.* 1988*b*). The reaction mixture was preincubated at 25°C for 5 min and contained [γ-^{32}P]GTP (0·1 μCi/assay), 2 mM-$MgCl_2$, 0·1 mM-EGTA, 0·2 mM-AppNHp, 0·1 mM-ATPγS, 10 mM-DTT, 5 mM-creatine phosphate (Tris salt), 0·4 mg ml^{-1} creatine kinase and 2 mg ml^{-1} BSA in 50 mM-triethanolamine·HCl, pH 7·4, in a total volume of 100 μl. The reaction was initiated by the addition of 30 μl of membranes to 70 μl of the reaction mixture and conducted for 3 min. The reaction was terminated by the addition of 0·5 ml sodium phosphate buffer (50 mM), pH 2·0, containing 5 % (w/v) activated charcoal. The reaction tubes were centrifuged for 5 min at 10 000 ***g*** at 4°C and the radioactivity of the supernatant was determined using Čerenkov radiation.

Reconstitution assay

D. discoideum and *synag 7* cells were grown as described above. Cells were resuspended to 1×10^8 cells ml^{-1} at 0°C in 10 mM-Tris·HCl, pH 8·0, 1 mM-$MgSO_4$, 0·2 mM-EDTA, 200 mM-sucrose, and lysed by pressing through a Nuclepore filter. The lysate was centrifuged at 10 000 ***g*** for 10 min. The supernatant was removed, centrifuged at 10 000 ***g*** for 10 min and used for reconstitution assays.

NC-4 and *synag 7* membranes were prepared as described above ('Culture conditions and membrane isolation'), washed and resuspended in the lysis buffer: 10 mM-Tris·HCl, pH 8·0, 1 mM-$MgSO_4$ to a final density equivalent to 10^8 cells ml^{-1}.

For reconstitution of GTP-binding, a mixture of supernatant and membranes (1:1) was incubated at 0°C for 10 min, then added (80 μl) to GTP binding mixture. After 2 min, incubation samples were centrifuged for 3 min at 10 000 ***g*** at 2–4°C, and the supernatant was aspirated. The pellet was dissolved in 80 μl 1 M-acetic acid, 1·2 ml Emulsifier was added and radioactivity was determined.

For reconstitution of GTPase, the supernatant and membrane mixture was washed after 10 min incubation, resuspended in 10 mM-triethanolamine·HCl, pH 7·4, to the same density and used in the GTPase assay.

Table 1. In vivo *responses to cyclic AMP in the* synag 7 *mutant*

Responses		Reference
Chemotaxis	+	This report
Cyclic AMP relay	−	Schaap *et al.* (1986)
Cyclic GMP response	+	Schaap *et al.* (1986)
Down-regulation	+	This report
Receptor modification	+	This report
GTP stimulation of adenylate cyclase	−*	Theibert & Devreotes (1986) Van Haastert *et al.* (1987)
GTP inhibition of adenylate cyclase	+	Van Haastert *et al.* (1987)

* GTP stimulation of adenylate cyclase is restored in *synag 7* lysates by the addition of a high-speed supernatant from wild-type cells (Theibert & Devreotes, 1986; Van Haastert *et al.* 1987).

Results

In vivo *responses to cyclic AMP*

The chemotactic response in mutant *synag 7* was slightly less sensitive to cyclic AMP than in wild-type cells, but in both cases the concentration of cyclic AMP that induced a response in 50% of the populations was between 10^{-9} and 10^{-8} M (Table 1). Because *synag 7* cells belong to different batches of cells, differences in their growing conditions may affect the chemotactic sensitivity to cyclic AMP. Therefore, we conclude that chemotaxis in *synag 7* is not significantly altered. Cells of *synag 7* had normal basal cyclic AMP levels (Schaap *et al.* 1986), but activation of adenylate cyclase by cyclic AMP *in vivo* was about 30-fold lower than in the parent strain NC-4 and was not improved when the cells were exposed to cyclic AMP pulses during starvation (Schaap *et al.* 1986). In mutant cells cyclic AMP-induced activation of guanylate cyclase was about 50% lower than in wild-type (Schaap *et al.* 1986). This response was restored when *synag 7* cells were pulsed with 10^{-7} M-cyclic AMP during differentiation (Table 1). Furthermore, treatment of mutant cells with 1 μM-cyclic AMP for 15 min induced loss of cyclic AMP binding to 10% of control values. This treatment of cells with cyclic AMP also induced the covalent modification of the receptor, which caused an increase in its apparent molecular weight from 40×10^3 to 43×10^3 M_r (Table 1).

Adenylate cyclase in the mutant could not be activated by cyclic AMP *in vivo* or by GTPγS *in vitro*. Inhibition of adenylate cyclase by GTPγS in the mutant membranes was not affected (Van Haastert *et al.* 1987). The wild-type pattern of adenylate cyclase regulation was restored in *synag 7* lysates by the addition of a high-speed supernatant from wild-type cells (Theibert & Devreotes, 1986; Van Haastert *et al.* 1987). This indicates that the defect of the mutant is probably located in a cytosolic cofactor that is required for guanine-nucleotide-mediated activation of adenylate cyclase in *D. discoideum*.

In this paper the characteristics of cell surface cyclic AMP receptors, G-protein, and the interaction between cyclic AMP receptors and G-protein are described.

Cell surface cyclic AMP receptors

The association of 30 nM-[^{3}H]cyclic AMP to aggregative wild-type and *synag 7* cells is shown in Fig. 1. Binding of cyclic AMP to both types of cells rapidly increases, reaching a maximum after about 6 s, and subsequently declines to an apparent equilibrium value approached at about 45–60 s. It has been demonstrated that this decrease in cyclic AMP binding appears to be due to an interconversion of high-affinity to low-affinity binding forms of the receptor (Van Haastert & De Wit, 1984*c*; Van Haastert, 1985; Van Haastert *et al.* 1986).

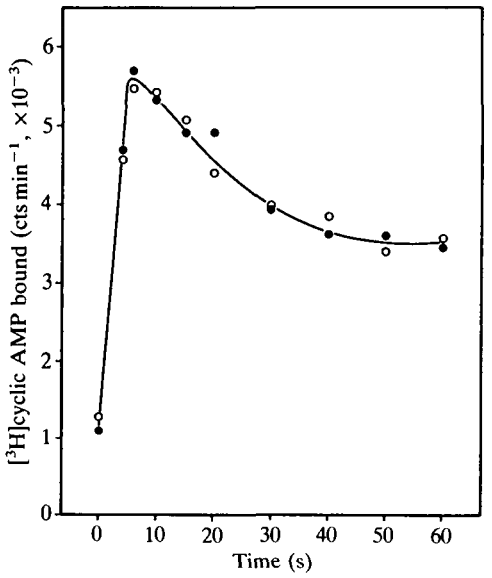

Fig. 1. Association of 30 nM-[^{3}H]cyclic AMP to wild-type (●) and *synag 7* mutant cells (○). Cells were incubated at 20°C with cyclic AMP and total cyclic AMP binding was measured at the times indicated. The results shown are the means of two experiments in triplicate, with intra-assay variation of less than 4% of the means.

The dissociation of the [^{3}H]cyclic AMP–receptor complex was measured to obtain more information on these binding types (Fig. 2). Wild-type and mutant cells were incubated with [^{3}H]cyclic AMP until equilibrium was reached, and then excess unlabelled cyclic AMP was added. The release of bound cyclic AMP from the mutant and wild-type receptor was identical. These types of experiment demonstrate that *D. discoideum* cells contain multiple forms of the receptor that have different affinities and rate constants of dissociation (Van Haastert & De Wit, 1984; Van Haastert, 1985; Van Haastert *et al.* 1986). These forms of receptor most probably reflect the interaction with G-protein (Van Haastert, 1984*b*; Van Haastert *et al.* 1986).

The inhibition of cyclic AMP binding by guanine nucleotides is shown in Fig. 3. Wild-type and *synag 7* membranes were incubated with 2 nM-[^{3}H]cyclic AMP to reach binding equilibrium in the absence and presence of GDPβS, GTPγS (Fig. 3), and GTP, GDP, GppNHp (data not shown). Binding of cyclic AMP was reduced to 35% in the presence of GTPγS and to about 55% in the presence of GDPβS (Fig. 3). The inhibition by GTPγS, GDPβS, GTP, GDP and GppNHp was essentially identical in wild-type and *synag 7* membranes. These results indicate that the *synag 7* mutant expresses normal

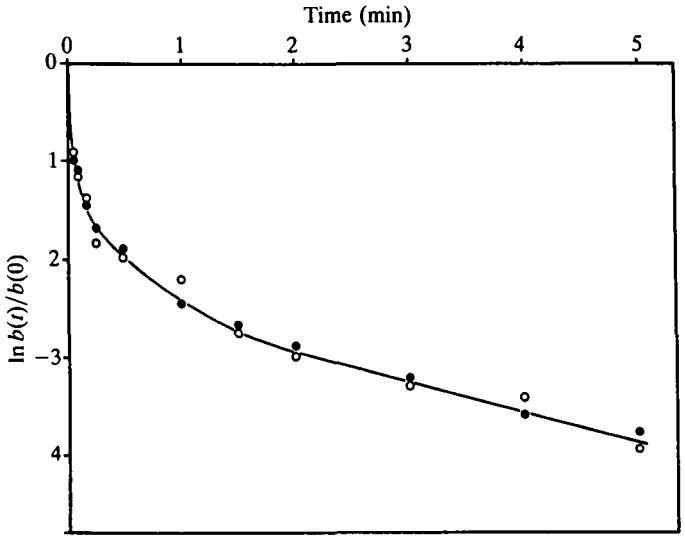

Fig. 2. Dissociation kinetics of [^{3}H]cyclic AMP–receptor complex. Wild-type (●) and mutant cells (○) were preincubated with 2 nM-[^{3}H]cyclic AMP for 45 s, and dissociation was induced at $t = 0$ by the addition of 0·1 mM-cyclic AMP. Binding was detected at the times indicated, $b(0)$ and $b(t)$. The means of two experiments are presented.

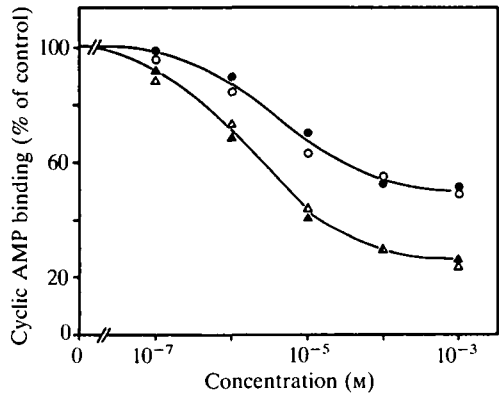

Fig. 3. Inhibition of the binding of 2 nM-[^{3}H]cyclic AMP to wild-type (●, ▲) and mutant (○, △) membranes by GDPβS (●, ○) and GTPγS (▲, △) after an incubation period of 75 s. The means of four experiments are shown with the error less than 10 % of the means.

levels of surface cyclic AMP receptor, which interact with G-protein as in the wild-type strain.

GTP binding

The kinetics of association of 100 nM-[^{3}H]GTP with wild-type and *synag 7* membranes are presented in Fig. 4. Binding equilibrium in wild-type membranes was reached within 60 s. Analysis of the association rate of [^{3}H]GTP binding (inset) to wild-type membranes indicated fast- and slow-binding types with half-times of association of about 4 s and 23 s, respectively. The binding at equilibrium was enhanced about 29 % by 10 μM-cyclic AMP without an obvious change in the association kinetics.

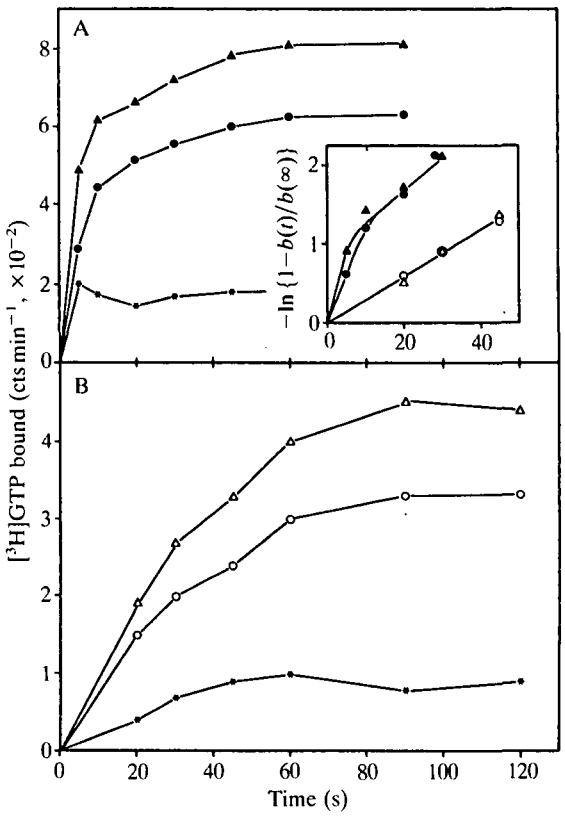

Fig. 4. Association of 100 nM-[^{3}H]GTP to wild-type (A) and mutant (B) membranes in the absence (●, ○) or presence (▲, △) of 10 μM-cyclic AMP. The results shown are the means of three experiments in triplicate, with intra-assay variation of less than 3 %. Inset: b_∞ equals the specific binding at equilibrium (90 s), b_t at t. The means of three experiments are presented, with the standard error less than 10 % of the means. The asterisks represent differences between (▲, △) and (●, ○).

The total [^{3}H]GTP binding to *synag 7* membranes was about 47 % lower than in control membranes and reached equilibrium after 90 s incubation (Fig. 4B). The fast-binding type was absent in the mutant membranes (inset, 4A). However, stimulation of GTP-binding to *synag 7* membranes by cyclic AMP had the same relative level as in wild-type membranes (33 %).

The kinetics of dissociation of bound [^{3}H]GTP are shown in Fig. 5. Wild-type and *synag 7* membranes were incubated with 100 nM-[^{3}H]GTP until equilibrium was reached and then excess unlabelled GTP was added. The release of bound [^{3}H]GTP was multiphasic, since a semi-logarithmic plot was non-linear. The rates of dissociation were essentially identical and accelerated by 10 μM-cyclic AMP in wild-type and mutant membranes.

For Scatchard analysis, wild-type and mutant membranes were incubated with different concentrations of [^{3}H]GTP and binding was measured at equilibrium (Fig. 6). The curve for [^{3}H]GTP binding to wild-type membranes was slightly convex, suggesting a complex binding process. These data confirm the complex kinetics of association and dissociation of [^{3}H]GTP (Fig. 5) and the recently described [^{35}S]GTPγS binding by two binding sites with, respectively, high ($K_d = 0{\cdot}2$ μM) and

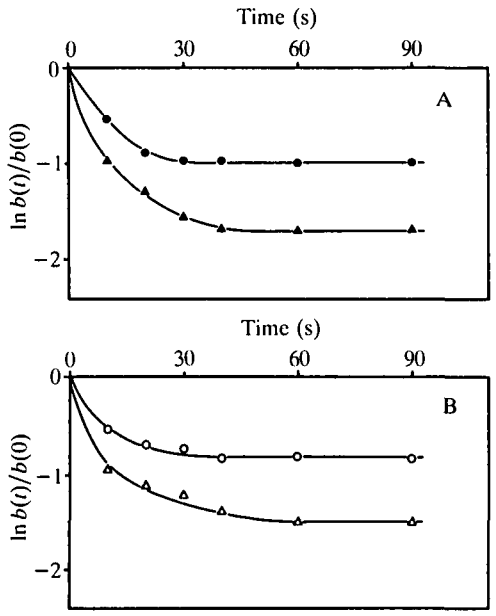

Fig. 5. Semi-logarithmic plot of dissociation of 100 nM-[^{3}H]GTP from membranes. Wild-type (A) and mutant (B) membranes were preincubated for 90 s with 100 nM-[^{3}H]GTP; then, at $t = 0$ rapidly mixed with 0·1 mM-GTP (●, ○) or 0·1 mM-GTP and 10 μM-cyclic AMP (▲, △). The results are the means of three experiments, with the standard error less than 10 % of the mean.

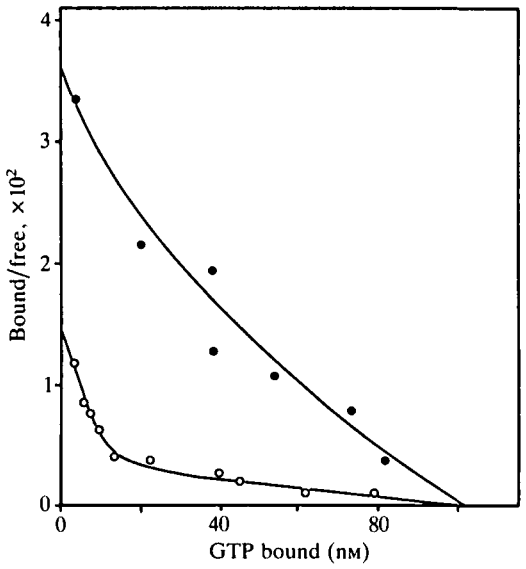

Fig. 6. Scatchard plot of [^{3}H]GTP binding to wild-type (●) and mutant (○) membranes after an incubation period of 60 s. The binding of different [^{3}H]GTP concentrations (0·9, 2, 5, 10, 20, 40, 80 μM) was measured. The results are the means of three experiments, with a variation of less than 10 % of the means.

Table 2. *Reconstitution of [^{3}H]GTP binding*

Conditions		GTP binding (%)
Membranes	Supernatant	
Wild-type	–	100
Wild-type	Wild-type	156·8 ± 11·2
Wild-type	*synag 7*	110·6 ± 8·6
Wild-type	Heated wild-type	100·0 ± 6·7
Wild-type	Heated *synag 7*	102·3 ± 9·3
synag 7	–	60·9 ± 2·4
synag 7	Wild-type	154·5 ± 13·4
synag 7	*synag 7*	71·3 ± 5·9
synag 7	Heated wild-type	60·0 ± 4·1
synag 7	Heated *synag 7*	61·2 ± 4·9

Membranes of wild-type and mutant *synag 7* were incubated at 0°C for 10 min with buffer, supernatant of wild-type or mutant strain, or supernatants heated at 60°C for 5 min. [^{3}H]GTP binding was determined in the membrane/supernatant mixture. The means of three experiments are presented.

low (K_d = 6·3 μM) affinity (Snaar-Jagalska *et al.* 1988*a*). However, the high- and low-affinity components for [^{3}H]GTP-binding do not differ much (apparent K_d of about 2 μM), making accurate calculations difficult; the total concentration of binding sites in wild-type membranes is 85 nM. Scatchard analysis in *synag 7* suggests that the [^{3}H]GTP binding to *synag 7* membranes is also heterogeneous. High- and low-affinity components can be easily deduced with apparent K_d values of 0·8 μM and 21 μM, respectively. The total number of binding sites in *synag 7* membranes was similar to that in wild-type membranes.

These observations suggest that the *synag 7* mutant has altered GTP binding. Reduction of equilibrium binding to mutant membranes is probably caused by loss of the fast-binding type. This defect does not disturb the signal-transduction pathway from cell surface cyclic AMP receptor to a putative G-protein, since cyclic AMP binding to mutant membranes is altered by guanine nucleotides and, alternatively, cyclic AMP increases [^{3}H]GTP binding to isolated membranes, at the same time accelerating the dissociation rate of GTP.

Reconstitution of [^{3}H]GTP binding

It has been shown that stimulation of adenylate cyclase by guanine nucleotides in *D. discoideum* membranes required a cytosolic factor. In mutant *synag 7* adenylate cyclase was activated by guanine nucleotide in the presence of a cytosolic factor from wild-type only, not from mutant cells (Theibert & Devreotes, 1986; Van Haastert *et al.* 1987).

Membranes of wild-type and mutant *synag 7* were incubated for 10 min at 0°C with PB buffer or supernatants isolated from wild-type or mutant lysates. Subsequently [^{3}H]GTP binding was determined at equilibrium (Table 2). In the presence of PB buffer [^{3}H]GTP binding to mutant membranes was about 40 % lower than to wild-type membranes. Supernatant of the mutant slightly (10 %) increased binding to both types of membranes. Addition of wild-type supernatant increased [^{3}H]GTP binding to wild-type membranes from 100 %

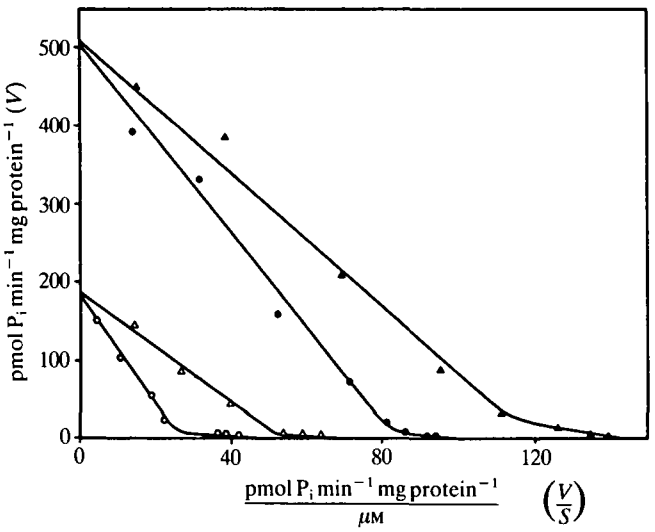

Fig. 7. Eadie–Hofstee plot of the GTP hydrolysis in wild-type and mutant membranes. Hydrolysis of [γ-^{32}P]GTP was determined at various concentrations in the absence (●, ○) or presence (▲, △) of 10 μM-cyclic AMP. Low-affinity GTPase was measured by addition of 100 μM-GTP and substracted from the total GTPase activity. The apparent K_m values (see text) of a high-affinity GTPase were extrapolated from the linear parts of the curve. The results are the means of three experiments, with the error less than 10% of the means. *V*, GTPase activity; *S*, GTP concentration.

to about 157%. Furthermore, wild-type supernatant restored [^{3}H]GTP binding to *synag 7* membranes (from 61% to 155%) to the same level. The component of the supernatant that restored GTP binding to *synag 7* was inactivated by heating to 60°C for 5 min.

These results suggest that the supernatant factor absent in *synag 7* mutant is essential for normal GTP binding.

GTPase activity and activation by cyclic AMP

It has been shown that GTP hydrolysis in *D. discoideum* membranes is caused by at least two enzymes with high and low affinity and that the high-affinity GTPase is stimulated by cyclic AMP (Snaar-Jagalska *et al.* 1988*b*). The hydrolysis of different concentrations of [γ-^{32}P]GTP by high-affinity GTPase in the absence and presence of 10 μM-cyclic AMP is shown in Fig. 7 as an Eadie–Hofstee plot. Basal activity in membranes of the *synag 7* mutant measured at 0·01 μM-GTP was about 55% lower than in wild-type membranes. However, the relative stimulation by 10 μM-cyclic AMP was only slightly lower in *synag 7* (44%) than in wild-type (50%). The stimulatory effect of receptor agonist on GTP hydrolysis by high-affinity GTPase occurred without a change in the V_{max} value and was apparently caused by an increase in enzyme affinity for GTP from 6·1 μM to 4·2 μM in wild-type and from 7·0 μM to 3·8 μM in mutant membranes.

Subsequently, the GTPase activity of the *synag 7* mutant was investigated under reconstitution conditions with added wild-type supernatant (Table 3). Because the supernatant contains high levels of non-specific nucleoside triphosphatase activity, membranes had to be

Table 3. *Hydrolysis of GTP in wild-type and* synag 7 *mutant by high-affinity GTPase*

Conditions		GTP hydrolysis (%)		
Membranes	Supernatant	−cyclic AMP	+cyclic AMP	Stimulation (%)
Wild-type	–	4·8 ± 0·3	7·1 ± 0·5	48 ± 14
Wild-type	Wild-type	4·7 ± 0·2	7·1 ± 0·5	51 ± 12
Wild-type	*synag 7*	4·8 ± 0·4	7·2 ± 0·3	50 ± 14
synag 7	–	2·1 ± 0·1	3·0 ± 0·3	43 ± 16
synag 7	Wild-type	3·4 ± 0·2	4·9 ± 0·2	44 ± 11
synag 7	*synag 7*	2·2 ± 0·1	3·1 ± 0·2	41 ± 10

Membranes of wild-type and *synag 7* were incubated at 0°C for 10 min in the presence or absence of supernatant, washed and used for GTPase assay. GTP hydrolysis was measured after 3-min incubation of membranes with 0·1 μM-GTP in the presence or absence of 10 μM-cyclic AMP. The results are means of three independent experiments. In all conditions stimulation by cyclic AMP was significantly above control values at $P < 0·01$; however, differences between % stimulation were not significant ($P > 0·5$).

washed after incubation with supernatant before they could be used in the GTPase assay. The supernatant of either wild-type or *synag 7* did not alter the GTPase activity in wild-type membranes. In contrast, the GTPase activity of *synag 7* was significantly ($P < 0·01$) increased by preincubation with wild-type supernatant, but not with the *synag 7* supernatant. It should be noted that, in contrast to GTP binding, GTPase activity in *synag 7* membranes is not completely restored by the wild-type supernatant; washing of the membranes and reversibility of the reconstitution could account for the partial recovery of GTPase activity in *synag 7* membranes.

Discussion

Mutants are valuable tools for investigating transmembrane signal transduction; therefore, the biochemical properties of the *D. discoideum* mutant *synag 7* were characterized. In this aggregation-defective mutant cyclic AMP and/or guanine nucleotides are not sufficient to activate adenylate cyclase (Theibert & Devreotes, 1986; Van Haastert *et al.* 1987). The wild-type pattern of guanine nucleotide regulation of adenylate cyclase is restored to the *synag 7* mutant by the addition of a high-speed supernatant from wild-type cells. Except for the activation of adenylate cyclase, cyclic AMP induces all responses in *synag 7* investigated to date (Table 1). *synag 7* cells express normal surface cyclic AMP receptors, which are regulated by guanine nucleotides as in wild-type cells. Cyclic AMP induces the down-regulation of the receptors, as well as their covalent modification. Cyclic AMP increases [^{3}H]GTP binding to mutant membranes, and at the same time accelerates the dissociation rate of bound GTP.

However, the association kinetics and equilibrium binding of [^{3}H]GTP are different if compared with those of wild-type membranes. In mutant membranes equilibrium [^{3}H]GTP binding is reduced and a fast-associating

component is absent. Also high-affinity GTPase activity is reduced but still stimulated by the receptor agonist. These results suggest that in mutant *synag 7* surface cyclic AMP receptors interact normally with G-protein. Considering that the coupling between receptors and adenylate cyclase involves the transduction of the signal *via* G-protein, the defect in the *synag 7* mutant must be localized at the point of interaction between G-protein and adenylate cyclase. Abnormal GTP binding and basal GTPase activity suggest that the defect is caused by an altered activation of G-protein or that the defect is localized at the point of interaction of an active GαGTP with basal adenylate cyclase. Addition of wild-type supernatant to *synag 7* membranes completely restored GTP binding and partly restored GTPase activities. These results suggest that this supernatant factor is essential for normal GTP binding, GTPase activity and activation of adenylate cyclase. The reconstitution occurred in the absence of detergent, which indicates that a soluble component is involved. This factor is heat-labile, and has a M_r greater than 20 000. It is unlikely that this factor is the stimulatory G-protein or Gsα, since stimulation of adenylate cyclase by GTPγS is associated with the membrane fraction and not with the cytosol fraction of a cell homogenate (Van Haastert *et al.* 1987). It is also unlikely that this factor represents the β subunit, because the β subunit is thought to serve as a membrane anchor (Spiegel, 1987). Preincubation of wild-type and mutant membranes under phosphorylation conditions converted stimulation of adenylate cyclase by GTPγS to inhibition. This inhibition was not affected by the cytosolic factor involved in stimulation of adenylate cyclase, but was abolished when membranes were obtained from cells treated with pertussis toxin (Van Haastert *et al.* 1987). These observations suggest that in wild-type and mutant cells an inhibitory G-protein in addition to a stimulatory Gs is present.

Recently a GTPase-activating protein (GAP) has been described in mammalian cells, which is required for optimal GTPase activity of *ras* proteins (Trahey & McCormick, 1987). The novel soluble heat-labile factor absent in the *synag 7* mutant shows functional homology with GAP, but the identity of these proteins has not been shown. It is tempting to suggest that the supernatant factor absent in mutant *synag 7* may belong to a family of proteins that regulate the function of G-proteins.

We thank Peter N. Devreotes for stimulating discussions. This work was supported by the Organisation for Fundamental Research (Medigon) and the C. and C. Huygens Fund, which are subsidized by the Netherlands Organisation for Scientific Research (NWO).

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(*Received 18 April 1988 – Accepted, in revised form, 15 July 1988*)